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Reliability of Serum Biomarkers of Inflammation from Repeated Measures in Healthy Individuals

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Abstract

Background—Biomarkers of low-grade systemic inflammation are used to study the associations of inflammation with chronic diseases, including cancer. However, relatively little is known about the intra-individual variability of most of these measures.

Methods—Fasting serum samples, collected at baseline and the end of ≥3 week washout periods in a 4-diet crossover feeding trial, were used to measure the inflammatory markers high sensitivity C-reactive protein (hsCRP), interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-8 and soluble TNF receptor (sTNFR) I and II. Participants included 62 men and women for analyses of IL-6 and CRP and 56 for analyses of IL-8, TNF-α, and sTNFRs, aged 20–40, who were free of factors known to influence inflammation, e.g., chronic disease, medication use, heavy alcohol use, smoking and obesity (body mass index >30 kg/m^2). Intraclass correlations (ICC) were estimated using random effects ANOVA, across all 4 time points (~6 weeks apart).

Results—ICCs for TNF-α and sTNFRI and II were very high: ICC=0.92, (95% CI: 0.89–0.96); 0.92, (95% CI: 0.88–0.95); and 0.90, (95% CI: 0.85–0.94), respectively. ICCs for IL-8 and hsCRP were 0.73 (95% CI: 0.63–0.83) and 0.62 (95% CI: 0.49–0.75), respectively. The ICC for IL-6 was considerably lower, ICC=0.48, (95% CI: 0.36–0.62). Three measures of IL-6 would be needed to achieve a reliability coefficient (Cronbach's alpha) of 0.75.

Conclusions—With the exception of IL-6, reliability of all inflammatory markers in our panel was high. Impact: This suggests that a single measure accurately captures the short-term (e.g., 4–6 months) variability within an individual.

Keywords

Intraclass correlation; reliability; inflammation; cytokines

INTRODUCTION

Cytokines, chemokines, acute phase proteins, and other soluble factors are involved in the inflammatory process, and cell survival, growth and proliferation (1). Circulating serum markers of inflammation are increasingly being used as biomarkers of low-grade systemic inflammation associated with risk of chronic diseases, such as rheumatoid arthritis (2), cardiovascular disease (3), and cancer (4). However, a relatively small number of studies have been published on the intra-individual variability (i.e., test-retest reliability) of most of these measures, with the exception of C-reactive protein (CRP) and interleukin (IL)-6, for

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which a large literature exists (5–17). The majority of existing reliability studies have examined samples at only two different time points, typically years apart. In addition, many of these studies are limited by small sample sizes or inclusion of participants with chronic conditions that may affect the inflammatory status of an individual. The primary aim of this investigation was to determine the intra-individual variability of six biomarkers of inflammation [i.e., high sensitivity (hs)CRP, IL-6, tumor necrosis factor alpha (TNF-α), IL-8, soluble tumor necrosis factor receptor (sTNFR) I and II] in serum across four time-points within a six-month time span, in a well-characterized population of healthy individuals. Results from this study will aid in selection of inflammatory biomarkers in intervention and population studies.

METHODS

Research Design and Study Participants

The study activities were conducted using archived samples from a completed study, "Enzyme Activation Trial 2" (2EAT), and carried out at Fred Hutchinson Cancer Research Center (FHCRC), Seattle, Washington, as described previously (18). The 2EAT study was a randomized, controlled, crossover feeding trial of Brassica and Apiaceous vegetables. Recruitment, enrollment, feeding, and sample collection, took place between March 2003 and July 2007; laboratory analysis for this project took place in 2010. Each diet was consumed for 14 days with a minimum of a 3-week washout period between the diets, for a total of 5–6 months participation in the study. Day 0 serum from each diet period was used for the present study. Eligibility criteria included men and women from the greater Seattle area who were healthy, and between the ages of 20 and 40 years. Exclusions were made for respondents with health conditions known to influence biotransformation enzyme activity and inflammation, such as chronic disease, medication use, heavy alcohol consumption, smoking, and obesity (body mass index >30 kg/m^2).

Analyses for 2 panels of inflammatory biomarkers, 1) hsCRP and IL-6; and 2) TNF-α, IL-8 and sTNFRI and II, were carried out separately. A total of 62 participants were included in the analyses for hsCRP and IL-6. Not all participants completed all time-points. The total number of participants per time point (T) was: n=62 for T1 and T2; n=59 for T3; and n=48 for T4. Samples were available for 56 individuals (27 men and 29 women) for analyses of IL-8, TNF-α, and sTNFR I and II. The total number of participants at each time point was: n=56 for T1; n=52 for T2; n=47 for T3; and n=25 for T4. The study was approved by the Institutional Review Board at the FHCRC and all participants gave informed consent.

Specimen Collection

Biological samples were collected during each 2-week feeding period at days 0 and 14 in the morning after a 12-hour overnight fast (19). Tubes without additive were allowed to clot at room temperature for 30 minutes before they were centrifuged to separate the serum. Serum was aliquoted and stored at −80oC. Day 0 serum collections for each diet period were used for the present study. The average length of time between sampling time points was 6 weeks.

Serum IL-6 concentrations were assayed using Quantikine high-sensitivity human IL-6 ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to manufacturer's instructions. The limit of quantification (LOQ) was 0.156 pg/ml. Serum hsCRP was measured using CRP Ultra Wide Range reagent (Genzyme Diagnostics, Framingham, MA) on a Roche Cobas Mira chemistry analyzer and read at 570 nm. The LOQ for this assay in our lab was 0.2 mg/l. IL-8 and TNF-α were multiplexed and assayed using the high-sensitivity Human Cytokine Panel, and the sTNFRs using the Human Soluble Cytokine Receptor Panel, (Millipore,

Billerica, MA). LOQs were 0.548 and 12.2 pg/ml, respectively. Samples were run in duplicate, and the median duplicate intra-assay coefficients of variation (CVs) were: 5.1% for IL-6; 5.9% for hsCRP; 6.3% for IL-8; 9.1% for TNF-α; 2.3% for sTNFRI; and 2.1% for sTNFRII. A blinded pooled serum sample was included in each batch to track plate-to-plate variation. The inter-plate CVs were 2.9% for IL-6; 3.1% for hsCRP; 15.3% for IL-8; 16.9% for TNF-α; 11% for sTNFRI; and 6% for sTNFRII. The assays were performed on never-thawed samples with the exception of hsCRP and the sTNFRs, for which once-thawed samples were used. All samples from the same individual for all diet periods were run on the same plate in duplicate. Other than 49 observations for hsCRP (of 248 total observations), none of the samples were below the limit of detection.

Samples were stored between 3 to 7 years prior to analyses. There was no indication that storage time was associated with degradation of any samples in adjusted regression models (partial R^2 ranged from 0.009–0.02 for all markers, data not shown). Health status was monitored using daily records which tracked, among other things, participant illness. If a participant reported illness before the start of a study period, they did not begin until the illness resolved; if the participant became ill during a study period, it was noted in the participant's chart.

Statistics

All inflammatory biomarker data were log-transformed prior to analysis to normalize distributions. Biomarker data are presented as geometric means and 95% confidence intervals (95% CI). A random effects analysis of variance model was used to estimate the intraclass correlations (ICC) and 95% CI across all 4 time points. We use the interpretation by Rosner (20), where ICC values between 0.4 to 0.75 indicate fair to good correlation, and values of 0.75 or greater indicate excellent correlation. To reduce the potential for inclusion of samples with unreported illness, outliers that fell 5 standard deviations (SD) above the median, and greater than a distance of 2 SD from any other data point, were entered as missing. For hsCRP values below 0.2 mg/l (20% of the observations, the majority from 11 individuals), multiple imputation using 10 imputed datasets with values between 0.01−0.19, with a mean and median of 0.1 was applied to estimate the ICC(21), and an adjusted standard error was used to calculate the 95% CI. For measures with an ICC below 0.5, we calculated the number of repeated measures that would be needed to yield a reliability (Cronbach's alpha) of ≥0.75. All analyses were performed using the StataSE, v12.0 (StataCorp, College Station, TX) statistical platform. All statistical tests are two-sided and a p-value <0.05 was considered statistically significant.

RESULTS

Table 1 gives the demographic characteristics and first blood draw (Time 1) serum concentrations of the inflammatory biomarkers for the study participants. Intraclass correlation coefficients using all 4 time periods are given in Table 2. Because we used diet periods for participants who did not complete all 4 diets, there are fewer observations at the third and fourth time-points, respectively. Intraclass correlation coefficients for TNF-α and the soluble TNF receptors I and II were excellent (ICC ≥0.90). The ICCs for IL-8 (ICC=0.73) and hsCRP (ICC=0.62) were good, whereas the reliability for IL-6 was fair (ICC=0.48). Three measures of IL-6 would need to be averaged to achieve a reliability coefficient (Cronbach's alpha) of 0.75.

DISCUSSION

In the present study, reliability of all inflammatory markers in the panel over 4 time points was good to excellent with the exception of IL-6, suggesting that, for these markers, a single measure accurately reflects an individuals' inflammatory status over a 4–6-month period.

Results for TNF-α (ICC=0.92) were higher than other studies reporting ICCs ranging from 0.48 to 0.69 in healthy individuals (7, 12, 15), although the interval in the present study was shorter, and time-points more frequent. Very few studies have evaluated the reliability of the soluble TNF receptors. Recently, Clendenen, et al (12) reported ICCs of 0.31 and 0.68 for sTNFRI and II, respectively, from 2 different time-points over a 2-year period in healthy women. However, the sample size was small (n=18) and the confidence intervals wide. Gu, et al (16) reported higher ICCs, 0.68 and 0.80 among 65 women, yet these values are still lower than the ICCs found in this study (>0.90). Reliability for hsCRP and IL-8 was good. Results for hsCRP are comparable with the reported ICCs in the range of 0.6–0.8 in various populations (5–11, 16). Investigations of the reliability of IL-8 have varied from fair to good (7, 12, 16, 17).

The reliability for IL-6 in this study was fair. This is similar to that reported by Ho et al (15), but lower than other studies (7, 14, 16, 17). It is not clear why this marker performed poorly relative to the other markers in this study, in terms of intra-individual reliability. All samples for the same individual were run in the same batch, on the same plate in duplicate, therefore assay variability was likely not a considerable factor. Because this was a healthy population with very low baseline inflammatory concentrations, we hypothesize that the large number of values near the bottom of the standard curve might be contributing to the lower reliability, as a smaller variance of exposure leads to lower reliability (22). It may also be that this marker is more easily perturbed by environmental exposures than other markers of inflammation. For example, physical activity acutely raises serum IL-6 more consistently than other inflammatory biomarkers and may be one source of variability (23, 24). IL-6 has been purported to play a crucial role in the pathogenesis of many chronic inflammatory diseases, and is frequently used as a measure of inflammation in many research settings, including those of autoimmune diseases, multiple sclerosis, inflammatory bowel diseases and many cancers (25). Therefore, further study of the reliability of this marker is warranted.

Advantages of this study include comparison of inflammatory biomarkers at 4 different time-points within a 6-month period, within the context of a randomized crossover design. Multiple fasting samples were obtained for the same individual, at the same time of day, with the same length of time between sample collections. The higher reliability of most of the markers in this study compared to other studies may reflect improved platform performance and sensitivity of the assays since previous investigations, or more frequent measures over a shorter duration than in previous studies.

Several limitations of the present analysis should be considered. First, although all observations in the present analysis were detectable, 21% of the samples for hsCRP were below an accurate limit of quantification (0.2 mg/l). These lower values of serum hsCRP are not unexpected given our healthy population. While this may have had a minor effect on the ICC, it has little clinical relevance as any value below 0.2 mg/l is extremely low and would be interpreted similarly. Of interest, other than low values for hsCRP, there were not any observations that were below the limit of quantitation for any of the other markers. Another limitation is the unbalanced distribution of samples by time point. Not all participants completed all 4 diet periods resulting in fewer participants at the third and fourth diet periods. Therefore, the power to detect reliability measures was reduced. Finally, although the stringent inclusion and exclusion criteria in the parent study provided for a homogenous

population and reduced potential confounding factors, this may have also limited the generalizability of the results. Further studies of longer duration, and in other populations, are needed.

Although long-term (e.g. year-to-year) reliability was not assessed, with the exception of IL-6, reliability of all inflammatory markers in this panel was high. For most markers, evidence is provided that a single measure accurately captures the month-to-month variability within an individual. These markers may be considered reliable for short-term population and intervention studies.

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References

1. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene. 1999; 18:6853–66. [PubMed: 10602461]
2. Waldburger JM, Firestein GS. Garden of therapeutic delights: new targets in rheumatic diseases. Arthritis Res Ther. 2009; 11:206. [PubMed: 19232066]
3. Klingenberg R, Hansson GK. Treating inflammation in atherosclerotic cardiovascular disease: emerging therapies. Eur Heart J. 2009; 30:2838–44. [PubMed: 19880848]
4. Aggarwal BB. Inflammation, a silent killer in cancer is not so silent! Curr Opin Pharmacol. 2009; 9:347–50. [PubMed: 19671496]
5. Ockene IS, Matthews CE, Rifai N, Ridker PM, Reed G, Stanek E. Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. Clin Chem. 2001; 47:444–50. [PubMed: 11238295]
6. Al-Delaimy WK, Jansen EH, Peeters PH, van der Laan JD, van Noord PA, Boshuizen HC, et al. Reliability of biomarkers of iron status, blood lipids, oxidative stress, vitamin D, C-reactive protein and fructosamine in two Dutch cohorts. Biomarkers. 2006; 11:370–82. [PubMed: 16908443]
7. Lee SA, Kallianpur A, Xiang YB, Wen W, Cai Q, Liu D, et al. Intra-individual variation of plasma adipokine levels and utility of single measurement of these biomarkers in population-based studies. Cancer Epidemiol Biomarkers Prev. 2007; 16:2464–70. [PubMed: 18006938]
8. Nasermoaddeli A, Sekine M, Kagamimori S. Intra-individual variability of high-sensitivity C-reactive protein: age-related variations over time in Japanese subjects. Circ J. 2006; 70:559–63. [PubMed: 16636490]
9. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med. 2004; 350:1387–97. [PubMed: 15070788]
10. Koenig W. Update on C-reactive protein as a risk marker in cardiovascular disease. Kidney Int Suppl. 2003:S58–61. [PubMed: 12694310]
11. Kayaba K, Ishikawa S, Gotoh T, Nago N, Kajii E, Nakamura Y, et al. Five-year intra-individual variability in C-reactive protein levels in a Japanese population-based study: the Jichi Medical School Cohort Study at Yamato, 1993–1998. Jpn Circ J. 2000; 64:303–8. [PubMed: 10783054]
12. Clendenen TV, Arslan AA, Lokshin AE, Idahl A, Hallmans G, Koenig KL, et al. Temporal reliability of cytokines and growth factors in EDTA plasma. BMC Res Notes. 2010; 3:302. [PubMed: 21073739]
13. de Maat MP, de Bart AC, Hennis BC, Meijer P, Havelaar AC, Mulder PG, et al. Interindividual and intraindividual variability in plasma fibrinogen, TPA antigen, PAI activity, and CRP in healthy, young volunteers and patients with angina pectoris. Arterioscler Thromb Vasc Biol. 1996; 16:1156–62. [PubMed: 8792769]

14. Cava F, Gonzalez C, Pascual MJ, Navajo JA, Gonzalez-Buitrago JM. Biological variation of interleukin 6 (IL-6) and soluble interleukin 2 receptor (sIL2R) in serum of healthy individuals. Cytokine. 2000; 12:1423–5. [PubMed: 10976007]
15. Ho GY, Xue XN, Burk RD, Kaplan RC, Cornell E, Cushman M. Variability of serum levels of tumor necrosis factor-alpha, interleukin 6, and soluble interleukin 6 receptor over 2 years in young women. Cytokine. 2005; 30:1–6. [PubMed: 15784406]
16. Gu Y, Zeleniuch-Jacquotte A, Linkov F, Koenig KL, Liu M, Velikokhatnaya L, et al. Reproducibility of serum cytokines and growth factors. Cytokine. 2009; 45:44–9. [PubMed: 19058974]
17. Hofmann JN, Yu K, Bagni RK, Lan Q, Rothman N, Purdue MP. Intra-individual variability over time in serum cytokine levels among participants in the prostate, lung, colorectal, and ovarian cancer screening Trial. Cytokine. 2011; 56:145–8. [PubMed: 21764327]
18. Navarro SL, Chang J, Peterson S, Chen C, King IB, Schwarz Y, et al. Modulation of human serum glutathione S-transferase-A1/2 concentration by cruciferous vegetables in a controlled feeding study is influenced by GSTM1 and GSTT1 genotypes. Cancer Epidemiol Biomarkers Prev. 2009; 18:2974–8. [PubMed: 19900941]
19. Navarro SL, Peterson S, Chen C, Makar KW, Schwarz Y, King IB, et al. Cruciferous vegetable feeding alters UGT1A1 activity: diet- and genotype-dependent changes in serum bilirubin in a controlled feeding trial. Cancer Prev Res. 2009; 2:345–52.
20. Rosner, BA., editor. Fundamentals of Biostatistics. 5. Pacific Grove: Thomas/Brooks Cole; 2000.
21. Rubin DB, Schenker N. Multiple imputation in health-care databases: an overview and some applications. Stat Med. 1991; 10:585–98. [PubMed: 2057657]
22. Armstrong, BK.; White, E.; Saracci, R. Principles of Exposure Measurement in Epidemiology. Oxford: Oxford University Press; 1994.
23. Drenth JP, Van Uum SH, Van Deuren M, Pesman GJ, Van der Ven-Jongekrijg J, Van der Meer JW. Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF-alpha and IL-1 beta production. J Appl Physiol. 1995; 79:1497–503. [PubMed: 8594005]
24. Pledge D, Grosset JF, Onambele-Pearson GL. Is there a morning-to-evening difference in the acute IL-6 and cortisol responses to resistance exercise? Cytokine. 2011; 55:318–23. [PubMed: 21632260]
25. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. Cytokine Growth Factor Rev. 2011; 22:83–9. [PubMed: 21377916]

Table 1

Characteristics of study participants

Demographics		
	n	**Mean (SD)**
Age (y)	62	30.4 (6.1)
BMI (kg/m^2)	62	24.2 (3.3)
		%
Female	31	50
Race		
Caucasian	37	60
Asian	20	31
Other	5	8
Inflammatory Biomarker		Geometric Mean (95% CI)[1]
TNF-α	56	3.0 (2.2, 4.0)
sTNFRI	56	953 (859, 1,058)
sTNFRII	56	4,153 (3,903, 4,419)
hsCRP	62	0.5 (0.3, 0.6)
IL-8	56	3.3 (2.8, 3,9)
IL-6	62	1.0 (0.8, 1.2)

[1]Geometric mean T1 serum concentrations; all concentrations are reported in units of pg/ml except for hsCRP which is reported as mg/l

Table 2

Intraclass correlation coefficients (95% CI) by serum inflammatory biomarker

Biomarker	*n**	ICC
TNF-α	52	0.92 (0.89–0.96)
sTNFRI	52	0.92 (0.88–0.95)
sTNFRII	52	0.90 (0.85–0.94)
hsCRP	62	0.62 (0.49–0.75)
IL-8	50	0.73 (0.63–0.83)
IL-6	62	0.48 (0.36–0.62)

*Number of participants that completed ≥2 time-points; Time-points corresponding with illness were excluded